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Effects of the SV40 Large T Antigen and EJ Ras Oncogene on Fibronectin Localization in Human Endometrial Cells as Viewed by Confocal Laser Scanning Microscopy

Key Words
Fibronectin
Confocal microscopy
Endometrial stromal cells, human
SV40 large T antigen
EJ ras oncogene

Abstract
We utilized confocal laser scanning microscopy to examine the localization of fibronectin deposition in cultures of human endometrial stromal cells. We found that fibronectin in normal human endometrial stromal cell cultures was both intracellular, ocurring in rough endoplasmic reticulum and in perinuclear regions, and extracellular, occurring diffusely over the entire cell surface. Endometrial stromal cells were transfected with a plasmid containing an origin-defective Simian Virus 40 (SV40) which codes for a temperature-sensitive large T antigen. When these cells were placed under temperature-restrictive conditions for large T-antigen function, they exhibited staining patterns similar to normal endometrial cells. Fibronectin deposition in cultures of partially or fully transformed endometrial cells was not intracellular as in normal cells, but was localized primarily between cells. Cells expressing the SV40 large T antigen deposited fibronectin mainly in parallel clumps between cells. Cells expressing both the SV40 large T antigen and the EJ ras oncogene, at high cell density, displayed networks of fibronectin arranged in matrix-like patterns between cells. The malignant cell line examined, sarcoma cells, also exhibited fibronectin networks between cells. Cell density affected fibronectin deposition in endometrial stromal cells expressing the EJ ras oncogene. At low density, cells expressing the SV40 large T antigen and the EJ ras oncogene displayed diffuse fibronectin patterns and, at high density, these cells formed colonies with networks of fibronectin between cells.

Introduction

Fibronectin (Fn) is an adhesive extracellular matrix (ECM) glycoprotein that surrounds and supports cells in embryonic and adult tissues [1, 2]. In recent years, Fn has emerged as a prototype adhesive ECM glycoprotein for the study of normal cell migration during embryogenesis and of abnormal cell migration in the adult, i.e. tumor invasion and metastasis. In many instances, Fn is present in early development in embryonic cells and must dimin-

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ish for differentiation to occur [3]. The presence or absence of Fn deposition around a given cell can determine whether that cell participates in migratory movements during development. The phenotype of a migrating cell in vitro appears to be one which lacks cell surface Fn [4].

Some transformed cells resemble differentiating embryonic cells, because many transformed cells lack cell surface Fn. Transformation and metastasis could be viewed as a recapitulation of embryonic Fn expression and cell translocation; however, there are many exceptions to the generalization that transformed cells do not express Fn. Many malignant cells lack cell surface Fn [4], but transformed cells that retain a Fn matrix are not uncommon [4–8]. Additionally, many malignant cells fail to deposit Fn into a surrounding insoluble matrix even if they can synthesize Fn [4]. The correlation between the lack of cell surface Fn and transformation is not straightforward.

The role of specific oncogenes and their protein products on Fn expression is an emerging and important field. Recently, it was reported that the expression of the Fn receptor complex was enhanced 4.7-fold in Rous sarcoma virus-induced tumors [9]. Since one of the properties of transformed cells involves a defect in signal transduction, and since the ras oncogene has been implicated in sustained signalling that may lead to abnormal proliferation [10], we questioned what effect this altered signal transduction had on Fn. In our investigation, we studied the effects on Fn localization of the SV40 large T antigen, which is identified with an establishment or immortalization function, and EJ ras, an oncogene acting on the plasma membrane with a p21 protein product.

Transfected cell strains utilized contained an origin-defective construct of the temperature-sensitive mutant A209 of Simian Virus 40 (SV40) large T antigen and a c-Ha ras oncogene (EJ ras) either alone or in combination. The SV40 large T antigen increases the lifespan of human cells in culture [11, 12]. The SV40 large T antigen was functional at 33 °C, the permissive temperature (Tp) for large T antigen function; at 39 °C, the nonpermissive temperature (Tnp), the SV40 large T antigen was nonfunctional. Some human endometrial stromal cells (HESC) expressing the tsSV40 construct were additionally transfected with a pSV2neo/EJ ras plasmid, in order to examine the effects of these cooperating oncogenes on Fn deposition and organization. By utilizing the temperature-sensitive construct of the SV40 large T antigen, we were able to create cell strains which express both SV40 large T antigen and EJ ras oncogene (SV40/Ras Tp) by placing double transfectants at 33 °C. We were also able to place the double transfectants at the Tnp for large T-antigen function and, thus, create cell strains expressing only the EJ ras oncogene (SV40/Ras Tnp).

In addition to the normal and partially transformed HESC, we examined a line of neonatal human foreskin fibroblasts (HUFF), and a line of malignant uterine sarcoma cells. In this manner, correlations could be made between Fn deposition in neonatal cells (HUFF), normal differentiated human cells (HESC) and premalignant (SV40 Tp, SV40/Ras Tp, SV40/Ras Tnp) or malignant cells (uterine sarcoma).

Materials and Methods

Tissue Culture

Human endometrial tissue was obtained from hysterectomy specimens with no evidence of intrinsic endometrial disease [13]. Endometrial tissue was digested in a 0.2% collagenase solution and the stromal cells were separated from glands by a series of centrifugations as previously described [14]. Monolayer cultures of stromal cells were maintained in a 1:1 mixture of Opti-MEM I (Gibco, Gaithersburg, Md.) with 2-mercaptoethanol and RPMI 1640 (Gibco) supplemented with 1% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 3% bovine calf serum (HyClone), 2 μg/ml insulin, 4 m*M* glutamine, 2 × nonessential amino acids (Gibco), 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 2 μg/ml amphotericin B (Sigma antibiotics, St. Louis, Mo.). The cells were routinely subcultured at a 1:4 split ratio, and the medium was changed twice weekly. Normal HESC can be passaged up to around 20 population doublings before they senesce. However, all experiments were performed on normal HESC at less than 6 populations doublings. Periodic assays for contamination by mycoplasma were negative [15]. Neonatal HUFF were kindly provided by Dr. Virginia A. Lightner (Duke University, Durham, N.C.) and grown in the stromal cell media described above.

Approximately 10^6 HESC were transfected with 100 μg/ml of plasmid DNA in Opti-MEM at 4.0 kV/cm^2 for 30 μs. Cells were transfected with a SV40 construct defective in the origin of replication and coding for a mutant temperature-sensitive large T antigen. Cells were held at confluence until colonies of morphologically altered cells appeared. These were subcultured using cloning rings. Alternatively, the entire population was subcultured until the untransfected cells senesced and were overgrown. HESC transfected with a plasmid containing the tsSV40 large T antigen were evaluated by Southern blot analysis. These cells had the transfected gene present in low copy number, possibly at a single integration site [16]. Subsequently, HESC expressing the tsSV40 large T antigen were maintained in culture at the permissive temperature of 33 °C. A portion of the population was then transfected with an activated c-Ha ras oncogene carried by the plasmid PSV2neo/EJ [17, 18] kindly provided by Dr. Marc Mass (USEPA, Research Triangle Park, N.C.). EJ ras-transfected cells were then isolated by G418 selection for the genetically linked neomycin-resistance gene and the selected cell clones were maintained in culture at the Tp (33 °C). These double transfectants at Tp or Tnp demonstrated expression of the EJ ras

Table 1. Fibronectin distribution in human stromal cells

Cell type	Temperature °C	Cell shape	Fibronectin distribution			
			perinuclear	cell surface	between cells	
					low density	high density
HUFF	37	spindly	xx	xxx	x	xx
HESC	37	flat/rhomboid	xxx	xx	o	o
tsSV40	39	flat/rhomboid	x	o	o	o
tsSV40/EJras	39	flat/round	xx	o	o	xxx
tsSV40	33	rhomboid	x	x	xx	xxx
tsSV40/EJras	33	round/rhomboid	x	o	o	xxx
Sarcoma	37	rhomboid	x	o	xxx	xxx

o = No staining; x = mild staining; xx = moderate staining; xxx = intense staining.

protein product when stained with an antibody specific for the p21 protein product of EJ ras, which is the substitution of valine for glycine at position 12. HESC transfected with the tsSV40 construct, but not the EJ ras oncogene, were virtually unstained with this antibody [19]. Preliminary results by Northern blot analysis indicated EJ ras expression in HESC transfected with the SV40 construct and EJ ras oncogene at Tp or Tnp; no EJ ras expression was detected in HESC transfected with the SV40 construct alone (unpublished results).

Immunocytochemistry

Cells were plated onto 4-well multitest Lab-Tek plastic slides (Miles Scientific, Naperville, Ill.) at a density of 20,000 cells per well. Each slide was 25 × 75 mm total area. HUFF, normal HESC, and sarcoma cells were maintained at 37 °C for 5–7 days prior to staining. Normal HESC (control) and HESC transfected with the tsSV40 construct with or without the EJ ras oncogene were maintained at the Tp (33 °C) for large T-antigen function for 3 days. One set of slides containing all three cell types was transferred to the Tnp (39 °C), while an identical set of slides was maintained at the Tp. Cells were permitted to grow under these conditions for 4 days. Fn staining of cell cultures was performed in the following manner. Cells were washed twice with phosphate-buffered saline (PBS) after removing the media, fixed in 3.7% formaldehyde in PBS for 10 min at room temperature and subsequently rinsed in PBS. The cells were then permeabilized for 7 min with acetone at – 20 °C, and air dried. Then the cells were incubated in 250 µl/well of monoclonal anti-human Fn (Sigma F-7387) at a 1:20 dilution for 1 h at 37 °C, washed in PBS, and incubated for a further 30 min at 37 °C with a 1:32 dilution of fluorescein isothiocyanate (FITC)-labelled sheep anti-mouse IgG (Sigma). Cells were washed with PBS, mounted in media consisting of 5% n-propylgallate and 0.25% diazobicyclooctane [20] in polyvinyl alcohol [21] and observed with a Zeiss 10 Confocal Laser Scanning Microscope at a magnification of × 1,260. The software used on the CLSM was Axioscan (Carl Zeiss) running on an IBM-AT compatible Matrox FP2000 single board computer with a Matrox 512 image capture and processing system. Confocal fluorescent images were obtained using an argon laser of 488-nm wavelength and a 520-nm-long pass barrier filter. Transmitted differential interference contrast (DIC) images were obtained by utilizing the argon laser described above and no filters. Hard copy images were produced by a Polaroid freeze frame video printer.

Results

Confocal laser scanning microscopy enabled us to examine the localization of Fn deposition in cultures of normal, transfected and malignant HESC. Fn was localized to the following sites: (a) intracellular, typically perinuclear; (b) punctate extracellular or diffuse extracellular, distributed over the dorsal cell surface; (c) between cells, also usually in contact with the cell surface, but concentrated at the lateral cell-cell junction rather than the dorsal surface, and (d) between cells in a crisscrossing pattern linking cells together into colonies. Table 1 is a summary of Fn localization by all cell types described below. Fn-staining patterns in normal HESC correspond to earlier studies [14]. Fn in the normal HESC cultures was localized exclusively to the cells, either cell surface or intracellular (fig. 1–3); the only staining of the ECM apart from the cell surface was cell surface extensions (fig. 1), which might be Fn fibrils attached to and radiating from the cell surface. Fn is conspicuous in intracellular perinuclear regions, and radiates from the cell center as bright spots which appear to be rough endoplasmic reticulum (RER; fig. 2). Normal HESC appear to make and package Fn since their RER stain for Fn. HUFF exhibit a more extensive ECM-staining pattern than normal HESC, and unlike normal HESC display strands of Fn between cells. Normal HESC display a diffuse Fn-staining pattern of the ECM, whereas HUFF display more punctate patterns. Fn stains intensely in ECM fibers along the long axis of the HUFF, and in intracellular perinuclear regions: strands of Fn between these spindle-shaped cells are abundant (fig. 12). Thus, HUFF cells have characteristics of both the normal and transformed cells we investigated in this study. These neonatal cells possess Fn in association with

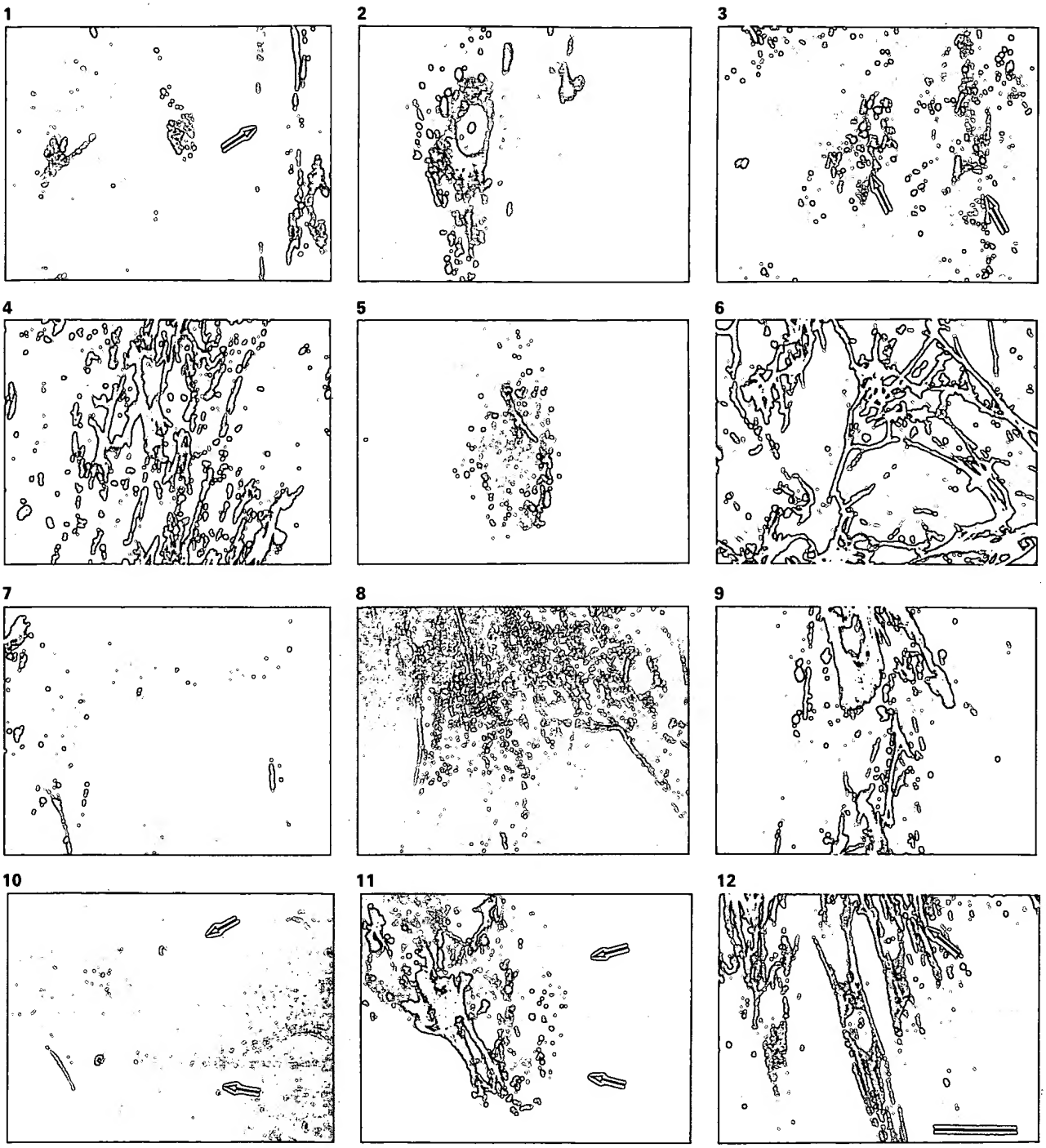


Fig. 1. Confocal micrograph of subconfluent cell culture of normal HESC (Passage, p, 2) at 33 °C. Perinuclear staining and scattered staining radiating from the cell center is obvious. Note Fn staining in cell surface extension (arrow). All micrographs are × 1,260.

Fig. 2. Subconfluent cell culture of normal HESC (p 5) at 37 °C. Perinuclear staining and RER which stain for Fn are apparent.

Fig. 3. Confluent cell culture of normal HESC (p 2) at 39 °C. Fn staining in RER and in perinuclear regions (arrows) is obvious. Fn was also diffusely distributed across the entire cell surface. Note that even when cells overlap one another as shown here, Fn does not appear between cells.

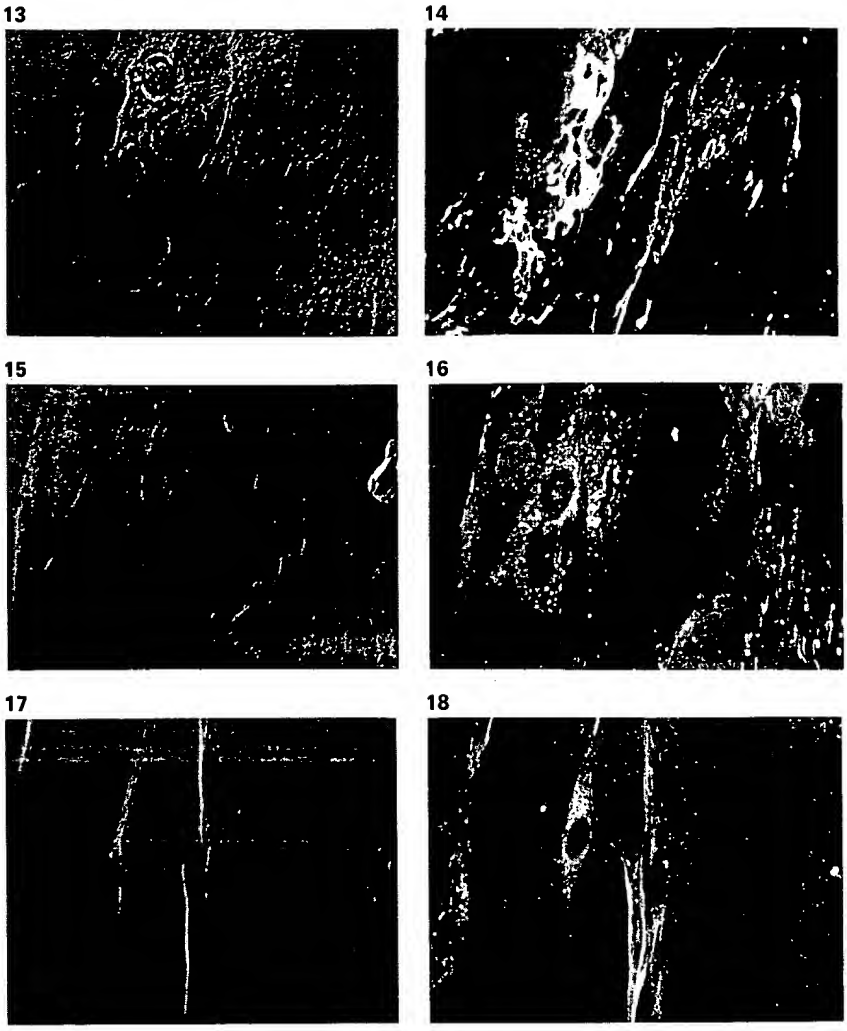


Fig. 13. DIC of confluent cell culture of SV40 (p 28) Tp.

Fig. 14. Confocal micrograph of cells shown in figure 13. Fn occurs at the lateral cell–cell junction in clumps mainly between cells.

Fig. 15. DIC of cell culture of SV40 (p 28) Tp.

Fig. 16. Confocal micrograph of cells shown in figure 15. Extracellularly, Fn is diffusely distributed across the cell surface and occurs as fibers or clumps between cells. Intracellularly, scattered RER stain for Fn and some perinuclear staining is observed.

Fig. 17. DIC of subconfluent cell culture of SV40/Ras (p 39) Tnp.

Fig. 18. Confocal micrograph of cells shown in figure 17. Intracellular Fn staining is intense and some Fn fibers occur between cells.

Fig. 4. Confluent cell culture of SV40 (p 28) Tp. Fn is deposited mainly between cells with a small amount of intracellular perinuclear staining.

Fig. 5. Subconfluent cell culture of SV40 (p 28) Tnp. Note perinuclear staining and RER radiating from the cell center.

Fig. 6. Sarcoma cell culture at confluence maintained at 37 °C. Fn occurs only between these cells and assumes a matrix-like morphology.

Fig. 7. Subconfluent SV40/Ras (p 42) Tp cell culture. An area of low cell density is shown. Cells display sparse Fn staining and scattered focal contacts which stain for Fn.

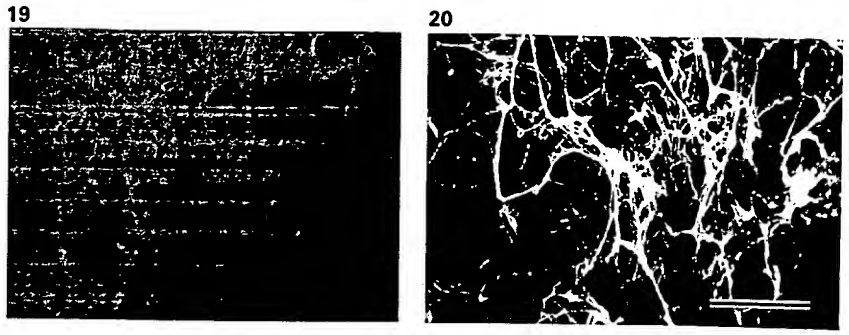
Fig. 8. DIC micrograph of a confluent SV40/Ras (p 42) Tp cell culture shown in figure 9. (Lines in DIC micrographs represent an artifact introduced by the detector.) Note that cells occur at high cell density and overlap one another.

Fig. 9. Confocal micrograph of cells shown in figure 8. This micrograph depicts Fn occurring between cells at high cell density.

Fig. 10. DIC micrograph of a SV40/Ras (p 42) Tnp cell culture shown in figure 11. Note that cell density is low (one large cell indicated by arrows) toward the right area of this micrograph, and cell distribution changes in the left region of this micrograph until cells overlap one another.

Fig. 11. Confocal micrograph of cells shown in figure 10. Fn-staining patterns vary dramatically depending on cell density. In the right area of the micrograph where cell density is low, one cell is seen with radiating parallel arrays of Fn from the cell center (arrows). The left area of the micrograph, where cell density is high, displays Fn forming an intricate network between the cells.

Fig. 12. Confluent HUFF cell culture at 37 °C. Fn is deposited in perinuclear regions and extracellularly over the entire cell surface. Fibers staining positively for Fn often run parallel to the long axis of the cell. Strands of Fn occur between these spindle-shaped cells (arrow). Bar 50 μm.



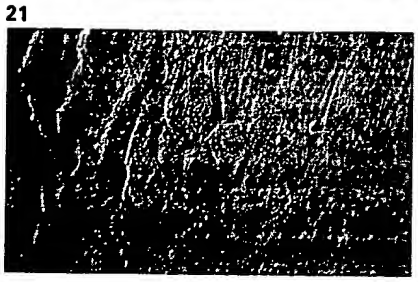


Fig. 19. DIC of confluent sarcoma cell culture shown in figure 20.

Fig. 20. Confocal micrograph of cells shown in figure 19. Note that Fn fibers form a matrix between cells. Bar 50 μm.

Fig. 21. DIC of a colony of SV40/Ras (p 38) Tp HESC. A fluorescence confocal optical sectioning series of these cells is shown in figures 22–25.

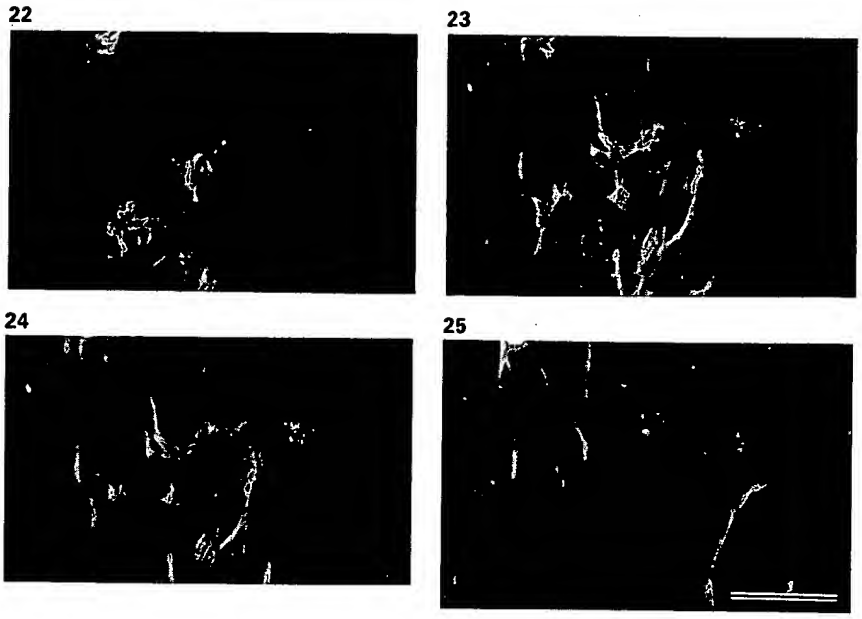


Fig. 22–25. Confocal optical sectioning series of colony shown in figure 21. Figures 22–25 are consecutive horizontal focal planes in which the colony was optically sectioned from an apical region of the colony to an area near the substrate. Fn is largely concentrated near the midplane of the colony as shown by figures 23 and 24. Figure 25 represents a section near the substrate which generally is out of the plane of focus for Fn staining, since Fn is concentrated several micrometers above this area. Two consecutive sections are 1.5 μm apart. Bar 50 μm.

the cell body as do normal HESC, but they also have strands of Fn between cells similar to the sarcoma cells examined.

HESC expressing the SV40 large T antigen (SV40 Tp) exhibit little perinuclear Fn staining but Fn staining is abundant between cells at lateral cell–cell borders (fig. 4, 13, 14). When cell density is high, Fn may occur in clumps between cells (fig. 4. 13, 14). Relatively little Fn is localized intracellularly in SV40 Tp cells, as compared to the intense staining in normal HESC. However, some scattered RER stain positively for Fn in SV40 Tp cells when cells occur at medium density (fig. 15, 16). When these transfected HESC are maintained at 39 °C (SV40 Tnp), the cells sometimes enlarge and flatten, and form sparse cultures (fig. 5). These cells exhibit bright spots radiating from the cell center, which appear to be RER, and intense

perinuclear staining (fig. 5, 17, 18). Fn may also occur between cells as fibers when cells are close together (fig. 17, 18). Thus, when the SV40 large T antigen is nonfunctional, Fn-staining patterns revert to a more intracellular location similar to the staining observed in normal HESC.

The addition of the EJ ras oncogene led to greater Fn localization between cells when cells occurred at high cell density. HESC expressing the SV40 large T antigen and EJ ras oncogene (SV40/Ras Tp) when cultured at low cell density often appear rounded and display sparse Fn deposition and Fn staining in scattered adhesion plaques (fig. 7). These cells exhibit the diffuse extracellular Fn staining seen in normal HESC, and some adhesion plaques stain positively for Fn. At high density, SV40/Ras cells at Tp form focal areas or colonies with extensive, fine networks of Fn between the cells (fig. 8, 9). Colonies of SV40/Ras Tp cells exhibit intricate crisscrossing networks of Fn between the cells (fig. 21–25). These networks are especially obvious at the midplane of the colony (fig. 23, 24). Only SV40/Ras Tp cells and malignant HESC (sarcoma cells) form colonies; normal HESC, SV40 Tp, and HUFF may line cells up in parallel at high cell density, but these cells do not overlap and form focal areas as do SV40/Ras Tp and sarcoma cells. SV40/Ras Tnp cells flatten and enlarge and display networks of Fn between the cells at high cell density (fig. 10, 11). This Fn pattern resembles SV40/Ras Tp at high cell density. At low cell density SV40/Ras Tnp cells possess parallel arrays of Fn which radiate from the cell center (fig. 10, 11). The Fn in the sparse cultures of SV40/Ras Tnp cells is prominent in cell surface association, but very little is present between cells. These cells, thus, resemble the untransfected HESC. However, cell–cell contact in the dense cultures of SV40/Ras Tp and Tnp cells appears to promote deposition of Fn into a matrix between the cells.

Sarcoma cells overlap one another and form colonies (fig. 19). Crisscrossing Fn fibers are obvious between cells (fig. 6, 20). Fn occurs in a matrix-like morphology between sarcoma cells, similar to the pattern seen for SV40/Ras Tp and Tnp at high cell density.

Discussion

We utilized the confocal laser scanning microscope to examine the distribution and localization of FITC-labeled Fn in cultures of normal, oncogene transfected and malignant HESC. One of the purposes of this study was to elucidate whether deposition or localization of Fn might be a suitable marker to study transformation of normal endometrial stromal cells. Using this approach we observed different arrangements of Fn in cultures of different cell types that spanned the spectrum of normal, transfected and malignant cells. Cell cultures of SV40 Tp, high-density cultures of SV40/Ras, and sarcoma cells exhibited Fn between the cells. In contrast, the Fn in normal HESC cultures was localized exclusively to the dorsal cell surface, in RER radiating from the cell center, and in perinuclear regions. In normal HESC, there was no staining of the ECM apart from the cell surface and, thus, there was no Fn deposition between the cells.

Normal HESC and HESC expressing the SV40 large T antigen display RER radiating from the cell center which stain positively for Fn. When these cells were stained for Fn without permeabilization by acetone, none of these Fn-positive RER were present, and only the cell surface stained positively for Fn (unpublished results). Fn perinuclear staining, presumably of the RER, has been described in mouse embryonal palatal mesenchymal cells [22] and in hepatocytes of rats with endotoxemia or sepsis [23]. In corneal endothelial cells, Fn was observed in RER cisternae where it was released 48 h after injury to be deposited in the cell Descement's membrane interface [24]. Tenascin also occurs in perinuclear RER in normal HESC, and like Fn is not incorporated into a matrix between cells [Vollmer et al., submitted].

Early studies of the effects of temperature-sensitive mutants such as Rous sarcoma virus (RSV) on Fn expression revealed that Fn was greatly reduced from the surface of RSV-transformed chick fibroblast cells compared to normal cells [25]. Further studies showed that transformation by RSV resulted in a generalized loss of extracellular matrix components [3]. Human fibroblasts transformed by SV40 had scant detectable surface Fn fibrils and a diffuse distribution of Fn receptors; addition of exogenous Fn restored the Fn matrix and receptor organization [26].

In contrast to these earlier studies, we observed that the expression of SV40 large T antigen had different effects on HESC Fn deposition. When HESC expressed the SV40 large T antigen, Fn was largely localized between cells at their lateral borders, but in association with the cell surface. When placed at the Tnp (i.e., SV40 Tnp), inhibition of the function of large T antigen was associated with a reversion of Fn localization to an intracellular, perinuclear site as was observed in normal HESC.

During embryogenesis, Fn expression in mesenchymal tissues appears to be correlated inversely with the process of differentiation. For example, Fn is present in early

development of embryonic cells such as mesenchymal chick kidney cells or developing mouse tooth germ, and must diminish for differentiation to occur [3]. Fn is found in undifferentiated mesenchymal tissue, but is lost when the mature collagen blastema is formed [3, 27]. Consistent with this view, the undifferentiated neonatal HUFF cells examined in our study displayed abundant Fn, whereas adult differentiated normal HESC had little Fn.

Previous findings suggest that Fn production should decrease as cells acquire a transformed and then an invasive, metastic phenotype. HESC expressing the SV40 large T antigen and the EJ ras oncogene have been shown to be invasive through a reconstituted human basement membrane, whereas HESC expressing only the SV40 large T antigen were not invasive [28]. Western blot analysis demonstrated that HESC expressing the SV40 large T antigen alone expressed more Fn and tenascin than did HESC cotransfected with SV40 plus the EJ ras oncogene [28]. HESC expressing the EJ ras oncogene deposited Fn between the cells, but only when the cells were maintained at high density. This occurred whether or not the SV40 large T antigen was expressed. This pattern is similar to the staining patterns seen in endometrial stromal sarcoma cells. There is little information regarding the involvement of Harvey ras, and Fn in the formation of primary endometrial sarcomas. However, several studies suggest that the level of expression and deposition of Fn may influence cell motility during tumor formation and metastasis. Anaplastic, nonmetastasizing prostatic tumors, and cell lines derived therefrom, exhibited a high metastatic potential after transfection with the v-Ha-ras oncogene. The level of Fn mRNA in metastasizing tumor cell sublines were 4- to 8-fold lower than in the nonmetastatic parental cell line [29]. Chinese hamster ovary (CHO) cells induced to overexpress the Fn receptor deposited more Fn in their ECM, and the cells migrated less than control CHO cells [30].

The switch in the level of expression and deposition of Fn may be important in primary tumor formation and the transition to a metastasizing cell. The idea that Fn must be present between cells during primary tumor formation and decrease for metastasis to occur is a recapitulation of the switch in Fn expression and deposition that occurs during embryogenesis. In our investigation, there was a change in Fn-staining patterns in the cells examined. In normal HESC, Fn occurred in an intracellular distribution. In HESC expressing the SV40 large T antigen, Fn was between cells at lateral cell–cell borders. In HESC expressing the EJ ras oncogene when cells were maintained at high cell density, Fn continued to occur between cells, but in a matrix-like pattern linking cells together. This latter staining pattern closely resembles the staining pattern seen in human uterine stromal sarcoma cells. From previous observations and the data reported here, we hypothesize that Fn is reexpressed during primary tumor formation. We believe that, early in the natural history of primary tumor formation, Fn functions to bind cells together, thus limiting their mobility. The acquisition of an invasive metastatic phenotype may be determined by the ability of primary tumor cells to gain the ability to degrade Fn [31], while retaining the ability to express but not lay down Fn, and retain the ability to respond to the mitogenic and chemotactic effects of Fn [31]. The tumor cells may appear to be recapitulating the process that is involved in embryogenesis, in which Fn is present during early development and must decrease for differentiation to occur.

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